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PRINCIPAL INVESTIGATOR: Brian Kavanagh

CONTRACTING ORGANIZATION: University of Colorado
Aurora, CO 80045

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Brian Kavanagh, Thomas Flaig

E-Mail: Brian.Kavanagh@ucdenver.edu

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14. ABSTRACT

The project was intended to determine whether cytotoxic ionizing radiation induces upregulation of Hif1-alpha via a nitric oxide(NO)-mediated tumor stress response pathway and whether this effect can be inhibited by the administration of dietary glycine supplementation, which can suppress activation of the macrophages responsible for the NO. The ultimate goal was to test whether glycine effects tumor growth delay after ionizing radiation by suppressing this pathway. PC-3 cells transfected with a Hif-1 reporter detectable via bioluminescence imaging were implanted into nude mice and subjected to ionizing radiation (2-6 Gy), with or without prior and concurrent feeding with a glycine-rich diet. The results established the feasibility of this experimental model and confirm an increase in Hif-1α expression after ionizing radiation. Furthermore, the administration of dietary glycine supplementation suppressed radiation-induced Hif-1α expression and created a favorable growth delay in the xenograft model.

15. SUBJECT TERMS
Prostate cancer, hif-1, glycine, nitric oxide, macrophage, radiation

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INTRODUCTION

The project is intended to determine whether a specific nitric oxide-mediated tumor stress response pathway that is initiated following a cytotoxic injury (1) is observed in prostate cancer xenografts, and (2) can be inhibited by the administration of dietary glycine supplementation. The rationale is based upon prior preclinical studies establishing, for other solid tumor types, that upregulation of inducible nitric oxide synthase (iNOS) in activated macrophages recruited to the site of cytotoxic injury from radiation or chemotherapy leads to the production of NO that stabilizes hypoxia-inducible factor 1-alpha (HIF-1α), which in turns leads to increased expression of vascular endothelial growth factor (VEGF) [Li 2007]. As a result of this signaling process, tumor angiogenesis is promoted, leading to recovery from the initial cytotoxic injury.

It is believed that inhibiting this NO-mediated process of angiogenesis can enhance the cytotoxicity of radiation or chemotherapy. Whereas glycine is known to inhibit the activation of macrophages by blocking a key chloride channel-mediated pathway [Rose 1999; Froh 2002], it is believed that glycine might be effective in abrogating the angiogenic response by thus indirectly preventing HIF-1α upregulation. The useful result of this signal modulation would be enhanced tumor growth delay following exposure to a cytotoxic agent such as ionizing radiation.

BODY

The tasks outlined in the approved statement of work are as listed below, followed by the research accomplishments for each task

Task 1. PREPARATION FOR XENOGRAFT STUDIES

1a. Preparation, submission, and review of animal protocol by institutional committee for review of animal studies

This sub-task was successfully accomplished, and the University of Colorado Animal Care and use Committee continues to monitor the animal protocol activities. A copy of the approved animal protocol has already been submitted but is available upon additional request if desired. The institutional reference number is 83511(02)1E, and the title is the same as the overall project title.

1b. Transfection of prostate cancer cell lines (PC3, LNCaP) with HIF-1α reporter gene (firefly luciferase-based); validation in vitro with bioluminescence methods

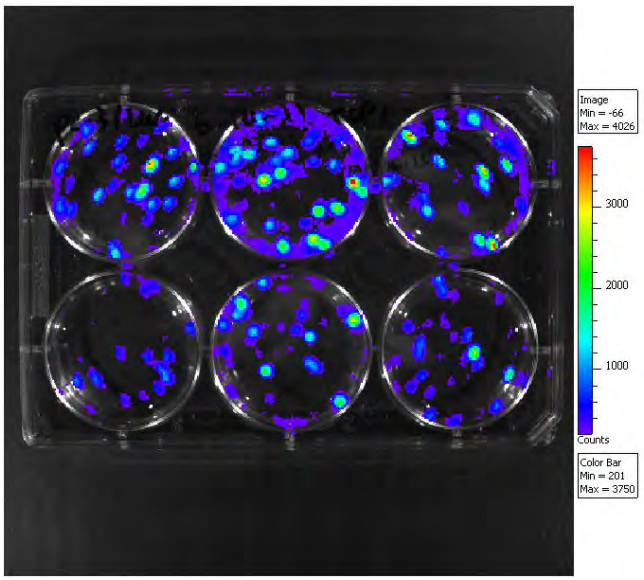
This sub-task has been successfully accomplished according to the methodology detailed below.

Cell Lines

The human prostate cancer PC3 and LNCaP cell lines were transfected (American Type Culture Collection, Manassas, VA, USA). All cells were grown in OptiMEM (Gibco, Grand Island, NY, USA) supplemented with 3.75% fetal bovine serum (Gemini, Woodland, CA, USA) and 100ug/ml streptomycin-100 IU/ML penicillin sulfate (Life Technologies, Grand Island, NY, USA). All cell lines were incubated at 37 °C with 5% CO2. Tumor growth rates from the PC3 line proved to be more suitable for the conduct of the intended experiments, and results from this cell line are the basis of this report.

Reporter Construct

A HIF-1-alpha inducible reporter construct was used as previously described (Moeller 2004). Cells were infected using a lentiviral vector and selected for using G418. Strongly luminescent clones were further selected and subcultured. To test the specificity of the reporter, cells were grown in normal conditions or given MG132, a proteosome inhibitor, for stress conditions at 10uM overnight and then imaged using the IVIS bioluminescense imager. After 1 minute of exposure luminescence was analyzed under each condition.



The figure to the right is a representative image of the in vitro growth and bioluminescence imaging of PC3 cells transfected with the HIF-1α reporter gene and stressed with MG132. The colonies with activity indicated on the associated color spectrum as trending toward the red side of the spectrum corresponded to cells that were successfully transfected. These colonies with high reporter uptake were selectively harvested and then expanded and maintained as stock in frozen form, from which aliquots

were thawed and re-expanded as needed for injection into the murine hosts for radiation experiments.

LNCAP cells were similarly successfully transfected (image not shown). However, as noted below, the LNCAP cell line proved less well suited for the xenograft studies as a result of the much slower in vitro and in vivo growth kinetics.

Task 2. XENOGRAFT STUDIES FOR SPECIFIC AIM 1: To determine whether radiation-induced cytotoxicity triggers a response in prostate cancer xenografts involving macrophage-mediated stabilization of Hif-1α and upregulation of VEGF

2a. Establish control growth rates for transfected cell lines

This sub-task has been successfully accomplished according to the methodology detailed below.

Tumor Implantation

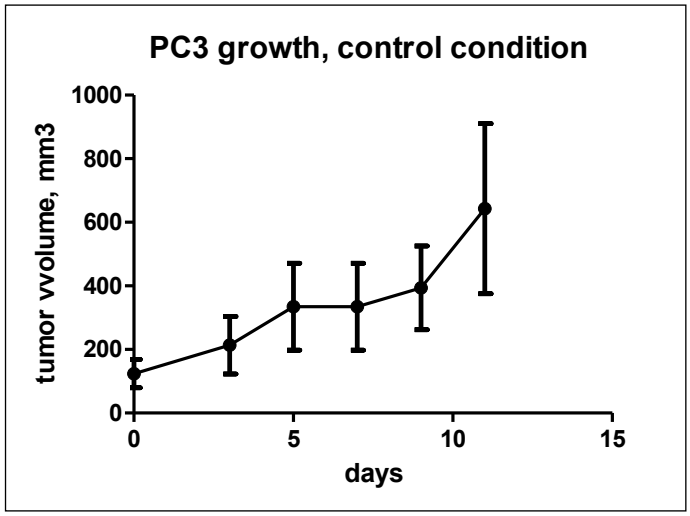
Xenograft tumors were implanted using the PC3-HIF and LNCaP-HIF cell lines. Growth velocity was ideal in the PC3 tumors and thus experiments utilized this cell line. Implantation was carried out in 6-8 week old athymic nude mice (National Cancer Institute). One and a half million cells/100uL PBS into the subcutaneous tissue on lateral hind leg of the mice. When working with the LNCaP-HIF line, 100uL of matrigel was used to stabilize cell growth conditions in vivo.

Tumor measurements

Experimental treatment began when tumors reached approximately 100 mm^3. Mice were then assigned to experimental groups with a consideration of equal average tumor volume per group. Tumor volume was calculated using length x width x depth.

Implanted transfected LnCAP cells required >4 weeks to grow to a palpable state, and this time frame did not appear feasible relative to the overall project. Thus, efforts were concentrated upon PC3 cells.

A representative growth curve after implantation of 10^6 PC3 cells in matrigel into the flank of the host animals (nude mice) is shown in the figure to the right, with measurements taken beginning with the first day when tumors were palpable, in this experiment approximately 30 days after implantation. Thus, in the graph shown, day 0 is the day at which tumors in a parallel cohort in the same experiment were irradiated, but here is shown a non-irradiated group.



Note that in this experiment and in all others, the staff veterinarian in the small animal facility evaluates the host animals frequently for their overall status and mandates sacrifice when the tumor growth has caused substantial distress to the animal. The maximum tumor volume reached before this assessment is generally in the range of 2000 mm^3 or smaller.

2b. For selected inoculum, irradiation (0, 2, 6 Gy) when palpable and bioluminescence imaging of HIF-1α

This sub-task has been successfully accomplished according to the methodology detailed below.

Radiation treatment

Mice were placed in a cesium (RS-2000 X Irradiator) radiation chamber for irradiation. They received 6Gy, 2Gy or sham irradiation (0 Gy). Mice were anesthetized with pentobarbital given intraperitoneally (IP) at 50mg/kg before irradiation. Only tumor was irradiated, and the remainder of the mouse was shielded with a custom lead shield.

Imaging

Luciferase expression was detected and quantified by using relative light units through the Xenogen IVIS200 imaging device and Living Image software (Xenogen, Alameda, CA). Mice were injected intraperitoneally (IP) with D-Luciferein Firefly (Xenogeny catalog XR-1001). Mice were imaged on selected days with the IVIS 200 Imaging system (Xenogen Corp., Alameda, CA USA) with day 0 as the date of irradiation. In order to obtain a signal, 200 ul of Luciferine (15mg/ml) was injected 5 minutes prior to anesthetization. Mice were then anesthetized with inhaled isoflurane at 5% initially and 2.5% for maintenance and imaged for 1 second (as a visual record of the tumors) and 1 minute (for total HIF-1α counts). The imaging system includes custom image analysis software that allows quantification of the total photon counts, which is a marker of HIF-1α expression. The total number of counts detected after 1 minute exposure via bioluminescence assay. A representative example of one day's image in one of the experimental groups is shown in the figure to the right. Serial quantitative results for a complete experiment are shown below.

It was soon appreciated that it was necessary to distribute animals with implanted tumors into groups of approximately equal size to allow for proper longitudinal analysis.

2c. For optimal dose showing effect in 2b, study of macrophage depletion using carrageenan treatment: control vs irradiated vs carrageenan vs irradiated+carrageenan

Carrageenan treatment began 24 hours prior to irradation. Mice were injected with 2mg IP and the treatment was continued for one repeat. However, due to the macrophage depletion the mice developed skin infections and poor health requiring antibiotic intervention and some deaths.

A single carrageenan experiment was performed, whereby 4 animals received0, 3, or 6 Gy radiation dose after tumor implantation and growth to palpable status. The results clearly indicated that carrageenan effectively eliminated the increased Hif-1α expression observed after radiation in control conditions (data not shown). However, it was also readily apparent that the carrageenan also appeared to put the animals at risk for a generally compromised overall health condition, presumably as a result of susceptibility to infection as a result of immune system compromise.

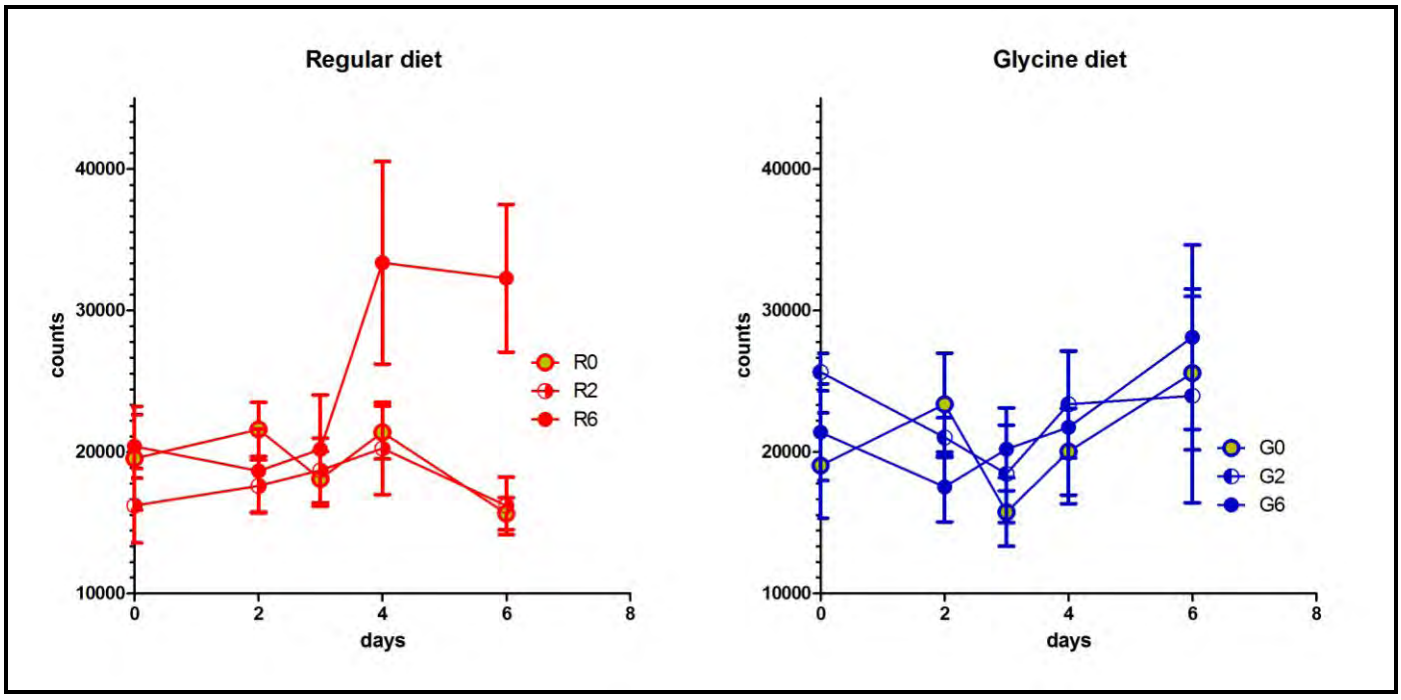
Task 3. XENOGRAFT STUDIES FOR SPECIFIC AIM 2: To determine whether inhibition of macrophage iNOS using a known iNOS inhibitor (L-NAME) or dietary glycine blocks radiation-mediated Hif-1α and enhances radiation-mediated tumor growth delay in prostate cancer xenografts.

This sub-task has been successfully accomplished according to the methodology detailed below.

3a. Bioluminescence imaging of HIF-1α: control v L-NAME v dietary glycine, all irradiated

Glycine therapy began 7 days (-7) prior to irradiation. Mice were changed from standard diet to a 5% glycine diet (Harlan Laboratories, Madison WI). Control diet was obtained from the same supplier. Diet change continued throughout therapy. L-NAME (500mg/L) therapy began at day -7 given in the drinking water. This change continued throughout therapy.

The raw data results of the experiment comparing glycine vs. regular diet are shown in the figure below:



R0, R2, R6 refer to animals who had a regular diet and were irradiated with 0,2, or 6 Gy, respectively, on day 0. G0, G2, and G6 are animals who had a glycine-supplemented diet and received the same range of radiation dose. There were 4 animals per group; results shown for each day of measurement are mean +/- SEM.

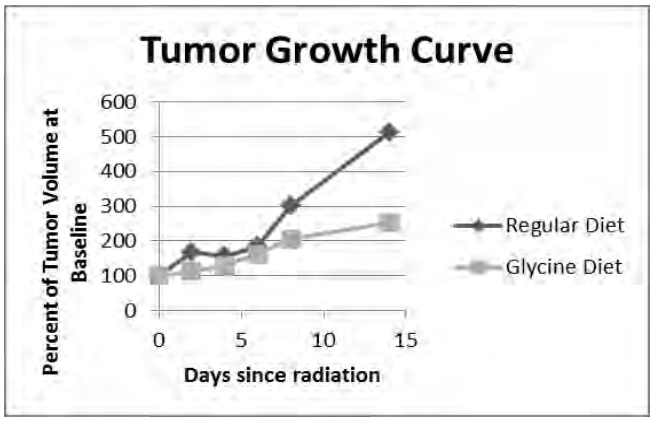
It may be observed in the graph on the left side that *HIF-1α* expression is significantly elevated on day 4 and day 6 ($p<0.05$ by t-test) following a dose of 6 Gy, but a dose of 2 Gy appears below the threshold to initiate an

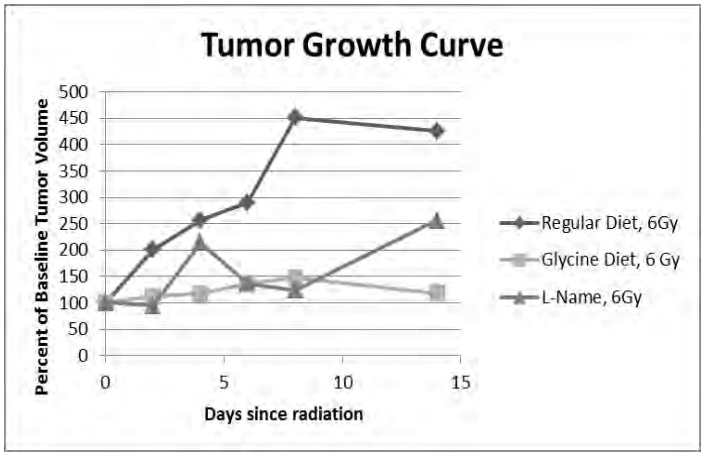
adequate amount of cytotoxicity to trigger macrophage infiltration. In the graph on the right, it may be observed that a glycine-supplemented diet effectively abrogates *HIF-1α* up-regulation. L-NAME effectively achieved similar

3b. Tumor growth delay: control vs glycine vs irradiation vs irradiation+glycine

This sub-task has been successfully accomplished.

The tumor growth curve for regular or glycine-supplemented diet groups without irradiation is shown to the right. Glycine treatment did appear to yield a modest growth delay in comparison to mice on regular diet. For conditions involving tumor irradiation to a dose of 6 Gy, results are shown in the figure below. Two issues are noteworthy. First, there is a suggestion that the 6 Gy irradiation actually causes faster tumor growth within 10 days than control conditions. It is possible that this phenomenon is a form of accelerated repopulation. Alternatively, another possible explanation is that inflammation and macrophage infiltration cause an artifactual enlargement of the tumors transiently after 6 Gy. More importantly, the second observation is that there is a tumor growth delay enhancement with either L-NAME or glycine-supplemented diet, consistent with the aforementioned suppression of radiation-induced *HIF-1α* upregulation. L-NAME is an established iNOS inhibitor.





KEY RESEARCH ACCOMPLISHMENTS:

- successful transfection of PC3 cells with Hif-1α bioluminescent reporter
- implantation of transefected cells into nude mice and characterization of growth rates under control conditions
- characterization of radiation-induced increase in Hif-1α expression in vivo in PC3 tumors
- abrogation of this increased Hif-1α expression with the use of carrageenan, establishing that the pathway likely involves macrophages
- abrogation of this increased Hif-1α expression with the use of either L-NAME or glycine-supplemented diet, demonstrating an effect on this macrophage-mediated pathway
- enhancement of radiation-induced tumor growth delay may be achieved with the use of a glycine-supplemented diet

REPORTABLE OUTCOMES:

- We plan to submit an abstract summarizing the findings above to the 2013 ASCO GU tumor symposium and/or next year's ASTRO meeting, and we will also submit a manuscript to the International J of Radiation Oncology, Biology, Physics or equivalent journal with a readership interested in this topic.

CONCLUSION:

The experiments performed support the feasibility of studying HIF-1α expression in vivo in prostate cancer xenografts. We established that there is an observable upregulation in HIF-1α expression after ionizing radiation, and this effect is abrogated with the use of a glycine-supplemented diet.

The inhibition of radiation-induced upregulation in HIF-1α expression corresponds to an enhancement of tumor growth delay. This result suggest a potential clinical application of glycine as a dietary supplement for patients being treated with radiotherapy for prostate cancer, and we are considering carefully various options regarding how best to translate the finding into a clinical investigation.

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